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ABSTRACT

Exposures to diesel exhaust (DE) and other petrochemical combustion products were the exposures reported by the greatest percentage of all Gulf War veterans (GWV). Along with diesel exhaust and other chemical exposures, psychological stress has been implicated in the onset of unexplained symptoms such as chemical sensitivity among GWV. The purpose of the proposed study is to test a model for chemical sensitivity in GWV, in which simultaneous acute exposures to DE and psychological stress cause increased symptoms via the acute phase response (APR), in susceptible individuals. Individuals who are low or high in the susceptibility factor of chemical intolerance (CI) will be exposed to DE either with or without a public speaking task, an acute psychological stressor. To date, 13 subjects have completed the protocol. The mean concentration in any single diesel exposure remained within ± 10% of the target PM mass concentration of 300µg/m3. Preliminary data indicates that relative to clean air subjects report a small increase in symptoms following the onset of diesel exposure. Analysis of blood cell counts and differentials reveal the reliability of the analytic techniques and compare favorably to normative reference ranges. Analysis of induced sputum cell differential counts show a high proportion of macrophages, verifying that the sputum originated in the airways. The percentages of neutrophils and macrophages are similar to reference values reported in other studies (Spanevello, et al. 2000). No hypothesis tests can be performed until a larger number of subjects complete the protocol.

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INTRODUCTION

Exposure to DE, kerosene, and/or other petrochemical vapors and incomplete combustion products was the exposure reported by the greatest percentage of all GWV (Kang et al., 2000) and was, along with numerous other self-reported exposures, associated with increased risk of various medically unexplained symptoms, including chemical sensitivity symptoms (Spencer, 2001; Fiedler et al. , Kipen et al., Wolfe et al., 2002). Many airborne chemical exposures may cause irritation of the eyes and respiratory tract, and recent controlled exposure studies have shown that DE (DE) can cause an acute inflammatory response in the respiratory tract. Such chemical exposure alone, however does not satisfactorily account for the multiple unexplained symptoms of veterans, and the stress of deployment and war has been cited as a contributory factor (Presidential Advisory Committee on GWV' Illnesses, 1996; Institute of Medicine). We are proposing to test a model for low-level chemical sensitivity in GWV (GWV's), in which simultaneous, acute exposure to DE and psychological stress cause symptoms through a common pathway, the acute phase response (APR). As demonstrated in experimental studies, the APR is activated independently by both psychological stress and local airway inflammation caused by acute inhalation of DE and other air contaminants. A main objective of the proposed study is to test the effects of an interaction between acute psychological stress and airway inflammation due to DE, an interaction that has not been studied previously. We will test several hypotheses suggested by this model, namely that: 1) Acute inhalation exposure to DE will cause a measurable local inflammatory response in the upper and lower respiratory tract, 2) Acute DE exposure alone will cause an APR, 3) An acute psychological stressor alone will cause an APR , 4) Simultaneous exposure to DE and an acute psychological stressor will interact additively or synergistically to enhance the APR, and 5) An enhanced APR will be associated with increased symptoms.

While exogenous exposures, such as DE and acute psychological stress, may well contribute to the symptoms of Gulf war illness, many veterans had no apparent health effects, suggesting that some individual psychological or physiological differences may have contributed to the response, and implicating some form of increased susceptibility. Studies of symptomatic GWV, however, cannot adequately test the interaction of susceptibility and exposure due to the potentially confounding effects of illness. Therefore, we also propose to test the effects of exposure to DE and stress among healthy subjects who are low or high in the susceptibility factor of self-reported chemical intolerance, a phenomenon of undefined mechanism.

BODY

Goals and Objectives for Year 2:

September, 2004 – August, 2005

A. Complete exposure sessions for 40 subjects

No subjects could participate in this research project until we received final IRB approval from the DOD and UMDNJ IRB. This approval was received in January of 2005. Since that time, 13 subjects were recruited to participate in pilot work to perform quality control for all aspects of our experimental protocol. Quality control procedures were established for subject exposures and for collection, processing, and analytical procedures of study endpoints to include blood immune markers, nasal lavage, and sputum samples. Following this initial pilot phase, a total of 21 subjects will have completed participation in the study through August 2005. Of these subjects, 13 subjects completed the protocol by July 31, and are included in preliminary analysis of outcomes data. We anticipate at least a continuation of this rate of subject participation with expected completion of the study by July of 2006.

Summary of subject participation to date:

PILOTS COMPLETED:		TOTAL
Induced Sputum Procedure		5
Protocol (No Exposure)		8
DROPPED OUT BEFORE PHYSICAL EXAM (Passed Screening):		
Parents Concerned	1	
Exposure/Cancer Concern	2	
Schedule Conflict	1	
		4
DROPPED OUT AFTER PHYSICAL EXAM:		
Parents Concerned		1
MEDICAL FAILURES:		
Difficult blood draw	2	
Childhood asthma	1	
Lab abnormalities	1	
		4

PARTICIPATE AT A LATER TIME: 1

REMOVED FROM PARTICIPATION:
Vasovagal 1

COMPLETED STUDY BY END OF JULY: 13 (Included in Analysis)

COMPLETE STUDY BY END OF AUGUST: 8

Development, implementation, and characterization of the diesel exhaust exposure system continued, and is summarized as follows:

1. Diesel Exhaust Delivery System:

The diesel exhaust delivery system was successfully installed for delivering diesel exhaust to the EOHSI Controlled Environmental Facility (CEF), as described in detail in last year's progress report. Since then, the system has been fine tuned and further characterized.

Major refining work includes the following. (1) A muffler was inserted between the exit of the engine tailpipe and the Animal Chamber Control Valve (see Fig1 showing the finalized system. Please note that the animal chamber is not used in the current study.) This custom-installed muffler is an URB 2 engine silencer from Cummins Inc. The insertion of the muffler has proven to be an effective way to reduce noise level within the CEF; and the current noise level during the engine operation is considered to be acceptable. (2) A valve lock was installed to fasten the delivery control valve, which helps reduce the vibration of the delivery pipe. (3) A second condensate bypass trap was added right before the diesel exhaust delivery pipe joins the dilution air pipe. This enables the removal of the condensables that would otherwise be deposited inside the delivery pipe (a difficult cleaning problem). (4) An integrated engine load module was developed to provide more stable and consistent engine load over the time. This load module, placed on a cart, consists of a fire extinguisher, 12 gauge wiring, four portable space heaters, an ammeter, and a circuit breaker box that accommodates four 20 Amp breakers. Each breaker controls one heater. By turning on one or more heaters at different power settings (low, medium, and high), the load module can provide an engine load ranging from 0% to 100% roughly at a 20% interval. The overall engine load (power output) is monitored with the ammeter (50 Amp, Model 1357, Simpson Inc.) because the output voltage is constant at 120 volt. A twist lock plug (50 Amp) and 10 gauge power cord are used to connect the load module and the generator.

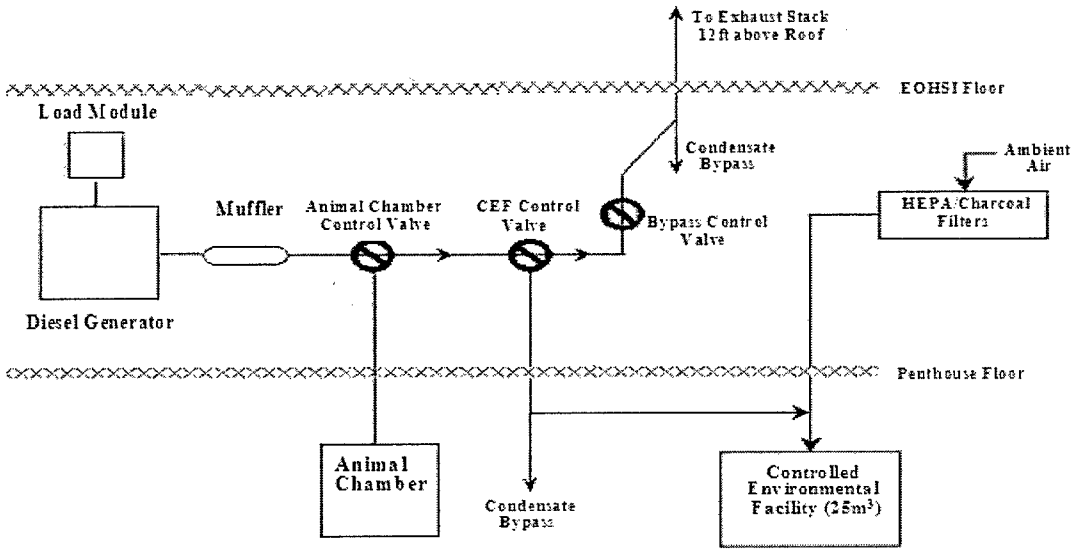


Fig 1: Schematic of the diesel exhaust system delivering diesel exhaust diluted with filtered ambient air to the Controlled Environmental Facility (CEF).

2. Diesel Fuel Analysis

The diesel fuel used for this study is stored in two 55-gallon drums at a specifically designated location according to the Rutgers University facility safety requirements. Based on our estimation, this amount of fuel (110 gallons) is sufficient for the entire project use. This prevents any variation in exposure conditions due to fuel composition variation. The properties of the fuel are analyzed by a certified laboratory, Caleb Brett (certificate No. 30701), in Carteret, New Jersey. The results are shown in Table 1.

Table 1. Specifications of premium diesel fuel, used in this study

Method	Test item	Results	Units
D4052	API Gravity	33.7	deg API
D86	Initial Boiling Point	364.5	°F
D4052	Density @ 15 °C	0.8561	g/ml
D445	Viscosity @ 100 °C	1.146	cSt
D613	Cetane Number	42.6	
D5291	Carbon	86.61	Wt%
D5291	Hydrogen	12.78	Wt%
D5291	Nitrogen	0.42	Wt%
D5291	Oxygen	0.19	Wt%
D4294	Sulfur	0.0446	Wt%
D1319	Aromatics	25.4	Vol%

3. Characterization of Diluted Diesel Exhaust

3.1 Particle Size distribution

As shown in last year's progress report, particles delivered to the CEF by the original EOHSI diesel exhaust delivery system are ultra fine and fine in size (diameter < 1 μm). After the system had been refined, we re-characterized the particle size distribution and chemical composition of the diluted diesel exhaust in the CEF. The results are summarized below.

Effects of Muffler on the Particle Size Distribution

The addition of the muffler has proven to result in a substantial reduction of the noise level. It also increases the traveling time of the diesel exhaust before reaching the CEF and, thus, may change the particle size distribution. The effect of muffler on the diesel exhaust particle was examined by comparison of particle size distributions before and after the installation of the engine muffler. The comparison results, as shown in Figure 2, indicate that the addition of the muffler reduced the number concentrations of particles with aerodynamic diameters smaller than 0.2 μm but slightly increased the number concentration of particles with aerodynamic diameters larger than 0.5 μm. However, the addition of the muffler did not appear to significantly change both mass-based and number-based size distributions (shapes of the curves shown in the figure).

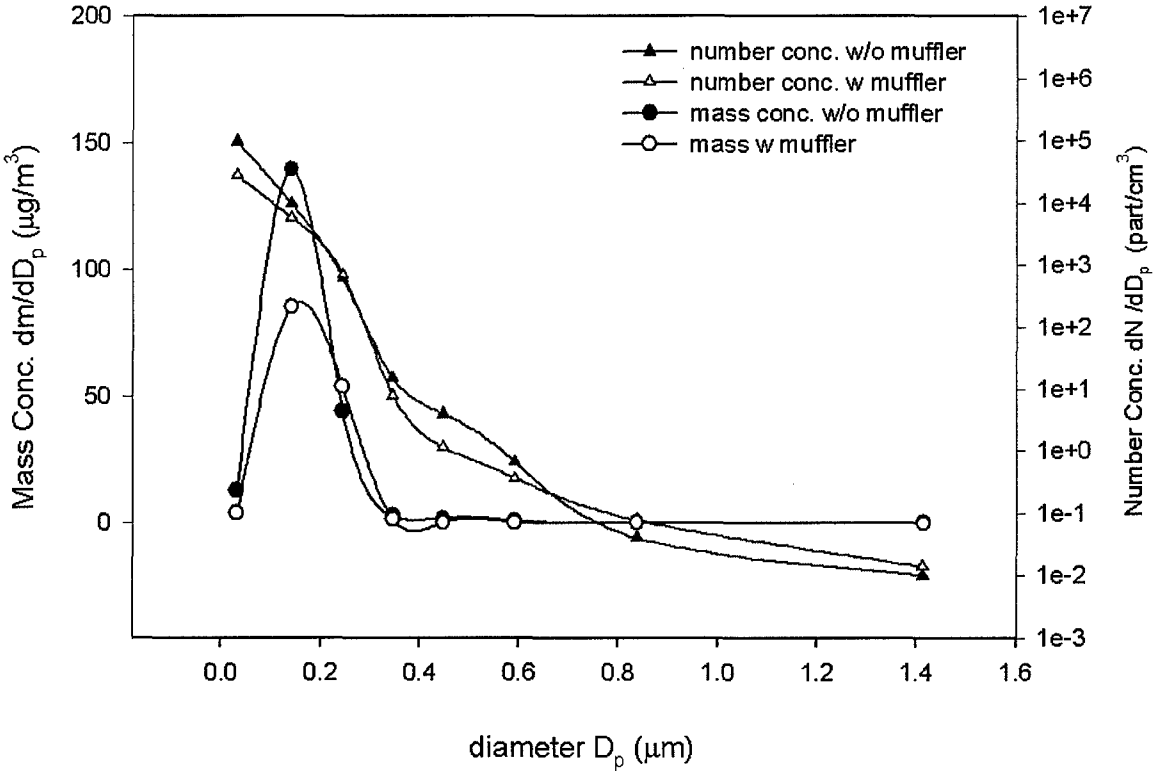


Fig 2: Effects of Muffler on Particle Mass and Number Distribution

Effects of Dilution Air Flow on Particle Size Distribution

Dilution conditions (e.g., relative humidity, temperature) play an important role in the nucleation and particle formation process (Kittelson, 1998), and, hence, influence the particle size distribution. In a set of experiments, CEF temperature and relative humidity were kept constant, but the flow rate of the dilution air was varied. Figure 3 shows particle size distributions under different flow rates, indicating that total number concentration decreased with a flow rate increase; and this effect was most profound for particles smaller than 0.1 μm. However, the size distributions (i.e., the shapes of the curves shown in Figure 3), both in terms of particle mass and in terms of particle number, remained the same. This observation is consistent with the fact that the size distribution of accumulation-mode aerosols is not sensitive to the dilution conditions (Kittelson, 1998). This is in favor of our health effects study because we can anticipate very similar size distributions of particles across all exposure sessions.

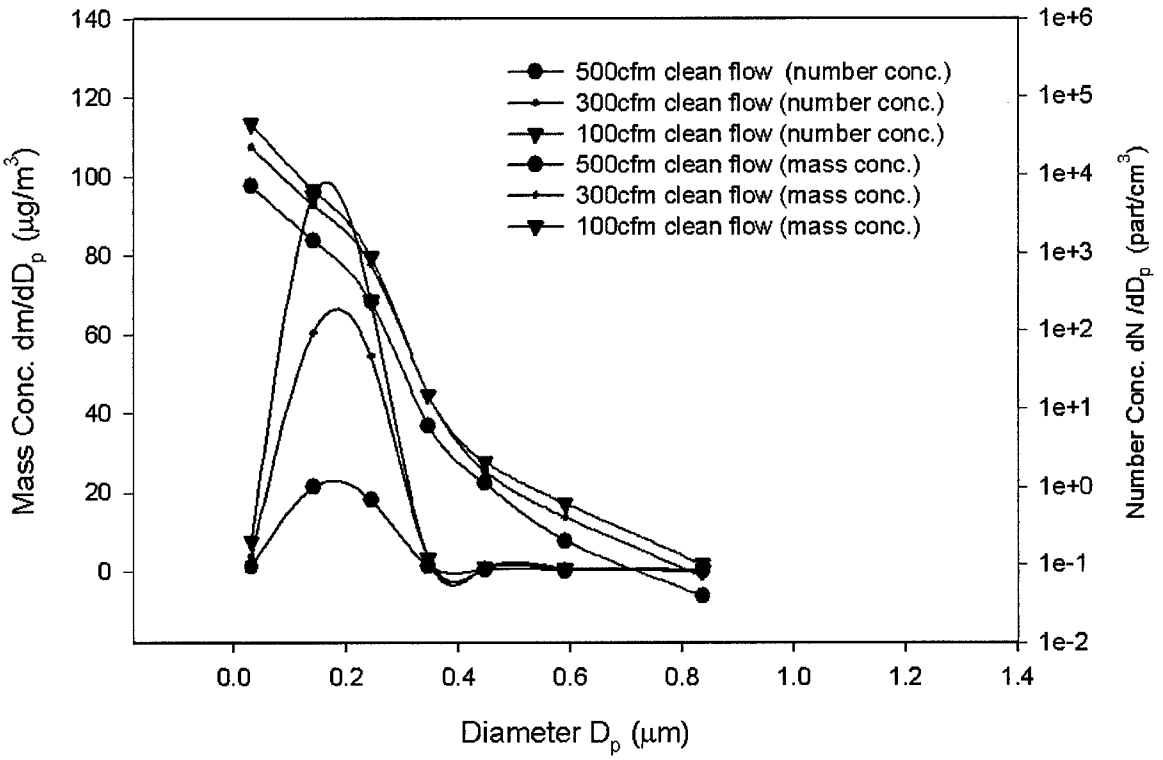


Fig 3: Particle Size Distribution with Different Dilution Air Flow

3.2.Chemical Composition

Several key diesel exhaust components were quantified, including $PM_{2.5}$ mass, nitric oxide (NO), oxides of nitrogen ($NO_x = NO + NO_2$), carbon monoxide (CO), elemental carbon

(EC) and organic carbon (OC) of particles, and a suite of aldehydes. (A suite of polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs are being analyzed; and the results will be available at a later date). The results are shown in Table 2. These results for our CEF conditions are compared with the results from another study in Lovelace Respiratory Research Institute, which used same engine model but different dilution conditions.

Previous studies (Shi et al., 2000) have shown that the CO concentration in raw exhaust is consistent, responding to different dilution ratios. Hence, CO is often used as an indicator of dilution ratio of the diesel exhaust. Table 2 shows that our dilution ratio was about 3 times that of the Lovelace system. The NOx/CO and PM/CO ratios from our system were in good agreement with those from the Lovelace system, suggesting that the two diesel exhaust delivery systems had similar gas and particle phase emission rates under the same engine load (100%). However, diesel exhaust particles in our system contained roughly equal amount of organic and elemental carbon; and total carbon accounted for 81% of PM mass. The EC/CO or OC/CO ratios for our system were different from those for the Lovelace system. Relative to dilution ratio, the aldehydes concentrations for our system were also different from those for the Lovelace system. The reasons for these differences may be complex and need further investigations.

Table 2: Chemical composition of diluted exhaust from the diesel generator operated at 100% loading. * adapted from McDonald et al., *Aerosol Science and Technology*, 2004.

Analyte	Units	EOHSI	Lovelace*
$PM_{2.5}$ Mass	$\mu g/m^3$	295	1032
NO	ppm	3.88	NA
NOx	ppm	4.04	12.0
CO	ppm	3.78	10.0
Formaldehyde	$\mu g/m^3$	324	229
Acetaldehyde	$\mu g/m^3$	143	67.1
Propionaldehyde	$\mu g/m^3$	22.3	72.4
Crotonaldehyde	$\mu g/m^3$	18.4	7.3
Hexaldehyde	$\mu g/m^3$	11.5	NA
Acetone	$\mu g/m^3$	2.32	10.5
Butyraldehyde	$\mu g/m^3$	12.2	NA
Benzaldehyde	$\mu g/m^3$	1.88	20.4
Valeraldehyde	$\mu g/m^3$	2.03	NA
Elemental Carbon	$\mu g/m^3$	115	660
Organic Carbon	$\mu g/m^3$	124	271

4. Exposure Conditions

Exposure sessions with human subjects commenced on June 6, 2005; and up to July 28, 2005, 13 diesel exhaust sessions and 13 clean air sessions have been completed. Each diesel exhaust session and each clean air session were monitored, using real-time monitors, for the

species and the parameters summarized in Table 3. Sample time-concentrations plots for the diesel exhaust sessions are shown in Fig. 4. It is typical that the steady state condition of diluted diesel exhaust was reached in the CEF within 15 minutes from the start of a session. The mean concentration in any single experiment remained within ± 10% of the target PM mass concentration of 300μg/m3.

Table 3. Summary of CEF exposure conditions for the sessions performed from June 6 and July 28, 2005

		PM mass conc. ($\mu g/m^3$)	PM number conc. ($\#/cm^3$)	NO conc. (ppm)	NO_2 conc. (ppm)	CO conc. (ppm)	Temp. (°C)	R.H. (%)
Diesel Exposure (N=13)	Mean	298	50959	1.831	0.071	2.73	22.5	40
	SD	25	14011	0.562	0.041	0.40	0.33	0.6
	Range	(267~334)	(32514 ~74208)	(0.929~ 2.71)	(0.66~0. 167)	(2.23~3. 31)	(22.1~2 2.9)	(39~ 41)
Clean Air (N=13)	Mean	15	4400	0.001	0.005	0.878	22.6	40
	SD	10	1964	0.0001	0.002	0.068	0.42	0.7
	Range	(4~31)	(2621 ~8614)	(0.0002 ~0.002)	(0.004~ 0.009)	(0.772~ 0.968)	(22.2~2 3.2)	(39~ 42)

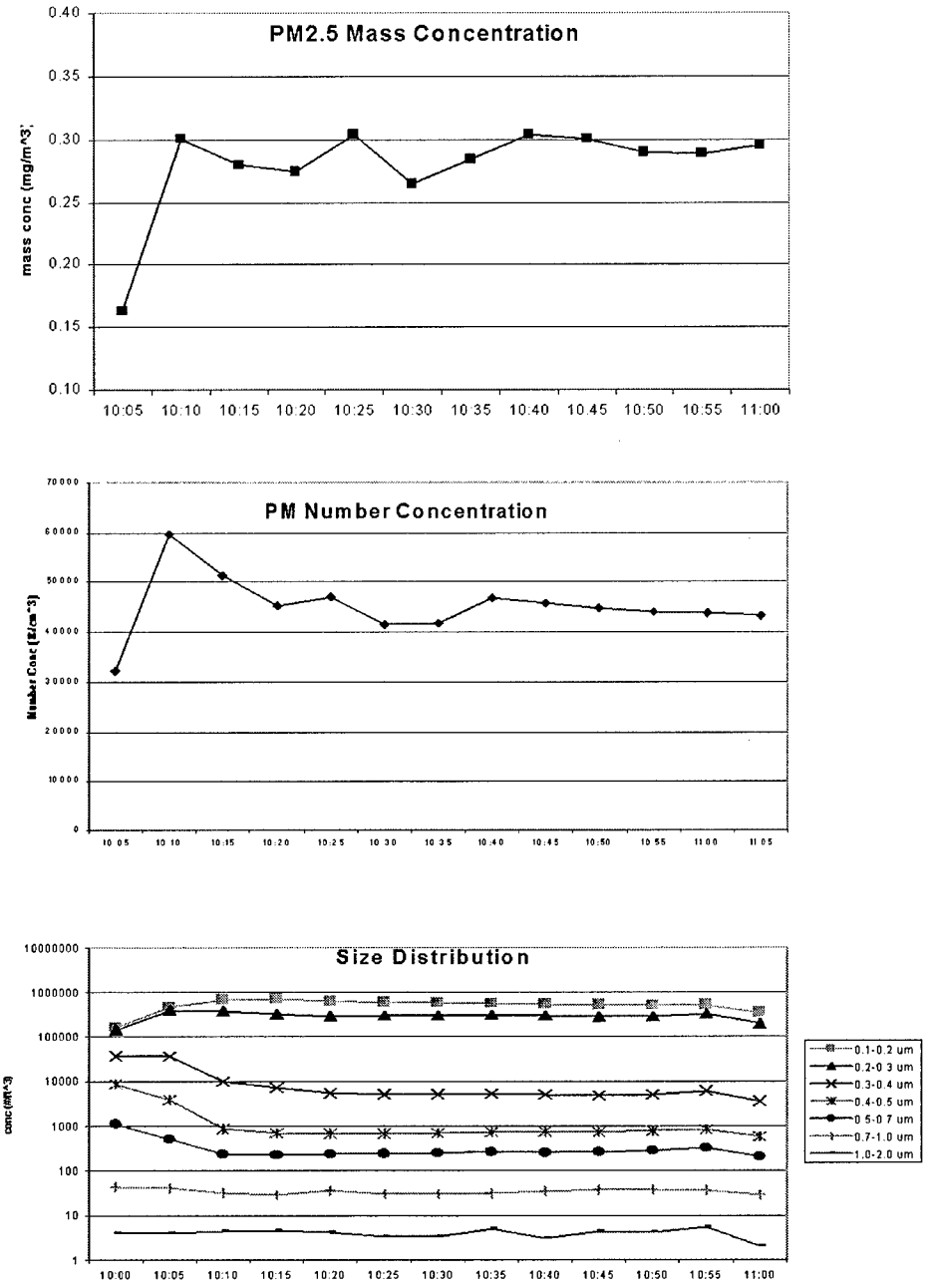


Fig. 4. PM mass concentrations, number concentrations, and size distributions vs. time of exposure

B. & C. Conduct data coding, and entry for 40 subjects; perform interim analysis of data.

Summary data for 13 subjects who completed the exposure protocol prior to July 31, 2005 are presented below. Table 4 gives the demographics for these subjects.

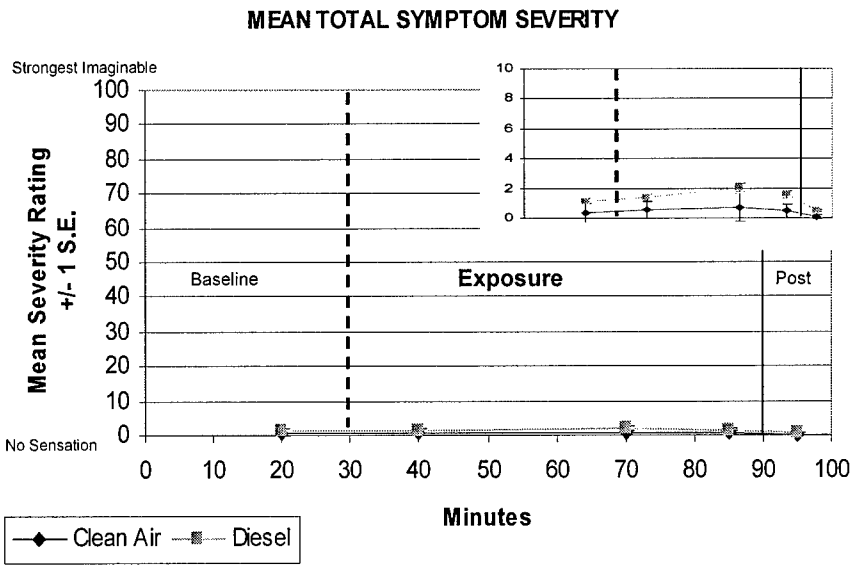
Table 4

DEMOGRAPHICS OF STUDY VOLUNTEERS (N=13)

	Females/Males
Gender	2/11
	Mean (S.D.)
Age	26.0 (5.6)
Years Education	17.8 (3.3)
Chemical Odor Intolerance Index	6.2 (2.0)
	Percent (N)
Race:	
Asian	39 (5)
White	31 (4)
Hispanic	23 (3)
Black	7 (1)

Symptom data for 13 subjects at time points before, during, and after exposure are reported in Figure 5. "Total symptom severity" represents aggregate mean symptom severity on a 100-point scale for 31 symptoms elicited by questionnaire. Additional symptom severity ratings at 6 hours and 24 hours post exposure have not been summarized yet. Results reported here show a modest increase in acute symptoms from the diesel exposure compared to the clean air control. Individual symptom means have not been examined to date, but are likely to show clearer effects for specific symptoms that have been associated with diesel exhaust exposure in previous studies (eg. eye irritation, respiratory tract irritation).

Figure 5



Blood cell counts and differentials (Flow cytometry)

Pilot analyses have been performed to affirm that our quality control procedures are working adequately for the laboratory analyses. Table 5 presents data for peripheral white blood cell counts, lymphocyte percentages, CD4 and CD8 subsets, and CD4/CD8 ratio from baseline (prior to exposure) blood collections. These results are within normal reference ranges reported. Comparison of data from baseline and exposure time points awaits completion of sufficient number of subjects to be meaningful.

Table 5

Pre-Exposure Blood (N=21)	Mean	Standard Deviation	Coefficient Variation	Median	Interquartile Range	Range
Lymphocytes (µl)	1789.98	543.53	30.36	1757.50	723.50	951.5-3275.0
Lymphocytes (%)	31.31	7.18	22.92	31.78	9.58	18.1- 43.0
Neutrophils (µl)	3622.00	1405.03	38.79	3797.00	1442.00	1710.0-8390.0
Neutrophils (%)	60.88	7.34	12.06	60.90	11.18	50.0- 73.9
CD_4 Lymphocytes (µl)	708.52	242.33	34.20	696.00	441.00	386.0-1129.0
CD_4 Lymphocytes (%)	39.68	6.58	16.58	40.78	5.58	23.8- 49.4
CD_8 Lymphocytes (µl)	420.19	234.92	55.91	404.00	316.00	175.0-1220.0
CD_8 Lymphocytes (%)	22.57	6.44	28.55	22.35	7.13	11.6- 37.3
CD_4/CD_8 Ratio	1.91	.57	29.80	1.87	0.49	0.6- 2.7

Induced sputum

During the pilot phase, the induced sputum procedure was performed with 5 subjects. Heavy saliva contamination of the sputum samples was observed. This was confirmed with differential cell counts showing significant (>50% of total cells) squamous epithelial cells. The procedure was modified to include isolation of sputum "plugs" from saliva using inspection and selection of plugs with an inverted microscope. The last 7 samples for which differential counts are available (through end of July 2005) show average epithelial cell contamination of <15%. Dilution of the sputum sample with saliva in earlier samples should not affect differential counts of nonsquamous cells (primarily PMNs and macrophages) which are reported below. In fact, whole sample (sputum and saliva) sputum analysis methods have been used successfully in a number of studies of environmental exposures. However, dilution of the sputum is likely to affect the concentration of soluble markers, such as cytokines, which are important outcome measures in this study. Analysis for these markers has not been performed to date.

As expected induced sputum cell differential counts show a high proportion of macrophages, verifying that the sputum originated in the airways (Table 6). The percentages of neutrophils and macrophages reported here are similar to reference values reported for 114 normal subjects (36 vs 27% and 62 vs 69%, respectively) (Spanevello, et al. 2000).

Table 6

Sputum Cell Counts (N=20)	Mean	Standard Deviation	Coefficient Variation
Total Cells (non-red) ($10^5 g^{-1}$)	23.1	22.1	95.5
Squamous Epithelial Cells ($10^5 g^{-1}$)	5.0	5.1	101.3
Squamous Epithelial Cells (%)	40.8	34.4	84.2
Neutrophils ($10^5 g^{-1}$)	8.7	18.5	212.1
Neutrophils (%)*	36.1	31.3	86.6
Macrophages ($10^5 g^{-1}$)	9.0	9.8	107.9
Macrophages (%)*	61.9	32.1	51.8

*Percent of non-squamous cells

C. Recruit 50 subjects for physical examinations and study screening to participate in the final year of the project.

We are in the process of actively recruiting and running subjects to complete the study by July of 2006, as planned. We are currently scheduling eight subjects per week and expect that, despite the original IRB delays, we will be able to complete the planned number of subjects by July of 2006.

KEY RESEARCH ACCOMPLISHMENTS

An abstract titled "The Effects of Diesel Exhaust and Stress on Systemic Inflammation and the Acute Phase Response" was submitted for presentation at the "Mechanisms of Action of Inhaled Fibers, Particles, and Nanoparticles in Lung and Cardiovascular Disease" conference, sponsored by NIEHS and NIOSH, on October 25-28, 2005.

REPORTABLE OUTCOMES

Given the small numbers of subjects who completed the study to date, tests of study hypotheses are not appropriate at this time. Analysis of outcomes and testing of hypotheses awaits completion of additional subjects. However, based on the development of our diesel exposure capabilities, the following projects have been initiated to augment outcome measures in the current study as well as additional studies using similar exposure paradigms and outcome measures.

Kipen, H (principal investigator). Responses to Fresh Aerosol in Susceptible Subjects, funded by EPA, R832144, $1,521,398.

The purpose of this study is to evaluate cardiovascular effects of diesel exhaust. Subjects will be exposed to 200 $\mu g/m^3$ $PM_{2.5}$ for 2 hours and platelet activation, endothelial dysfunction, and pulmonary inflammation through induced sputum will be measured.

"Noninvasive Measures of Oxidative Stress and Inflammatory Responses to Diesel Exhaust in Human Respiratory Epithelium." Laumbach RJ (PI). 12/2004-6/2005 Funded by NIOSH ERC Pilot project. $10,000. This study examines molecular markers of oxidative stress and inflammation in nasal respiratory epithelium samples from human subjects after controlled exposure to diesel exhaust. The goal is to develop and validate a new noninvasive techniques for studying responses to diesel exhaust in controlled exposure and epidemiology studies.

"Mechanisms of Responses to Diesel Exhaust and Stress." Laumbach RJ (PI). K08 Career Development, NIEHS. $139,137 x 5 years, Pending. This career award will provide support for development of the PI's capability to perform complementary studies of responses to diesel exhaust and stress in relevant animal models and human subjects.

CONCLUSIONS

Recruitment of subjects was significantly delayed due to deferral of review by the DOD IRB. Since IRB approval was received in January 2005, 13 pilot and 21 full protocol subjects have completed the study protocol. During the pilot phase, additional technical improvements were developed and implemented. We are presently recruiting at a rate of 8 subjects per week and anticipate that the study will be completed by July 2006. Additional exposure

characterization has demonstrated the reliability of our diesel exposure system. Based on preliminary review of data, techniques for measuring outcomes in induced sputum have been improved.

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